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# Presentation of Integrins on Leukocyte Microvilli: A Role for the Extracellular Domain in Determining Membrane Localization

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**Abstract.** Adhesion of blood leukocytes to the endothelium involves multiple steps including initial attachment (tethering), rolling, and firm arrest. Presentation of adhesion molecules on leukocyte microvilli can substantially enhance tethering. Localization of L-selectin to microvilli and of CD44 to the planar cell body have been shown to depend upon their transmembrane and cytoplasmic domains. We investigated the role of leukocyte integrin transmembrane and cytoplasmic domains in initiating adhesion under flow and in microvillous localization. Integrins  $\alpha 4\beta 7$ ,  $\alpha L\beta 2$ , and  $\alpha M\beta 2$  were heterologously expressed in K562 cells.  $\alpha 4\beta 7$  initiated adhesion under flow and localized to microvilli, whereas  $\beta 2$  integrins did not initiate adhesion and localized to the cell body. Chimeric integrins were produced by replacing the  $\alpha 4\beta 7$  cytoplasmic and/or transmembrane domains with the homologous domains of

$\alpha L\beta 2$  or  $\alpha M\beta 2$ . Unexpectedly, these chimeras efficiently mediated adhesion to the  $\alpha 4\beta 7$  ligand mucosal addressin cell adhesion molecule-1 under flow and localized to microvilli. Therefore, differences between the transmembrane and cytoplasmic domains of  $\alpha 4$  and  $\beta 2$  integrins do not account for differences in ability to support attachment under flow or in membrane localization. Integrins  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha E\beta 7$  also localized to microvilli. Transmembrane proteins known or suspected to associate with extracellular domains of microvillous integrins, including tetraspans and CD47, were concentrated on microvilli as well. These findings suggest that interactions between the extracellular domains of integrins and associated proteins could direct the assembly of multimolecular complexes on leukocyte microvilli.

LEUKOCYTE recruitment to tissues from blood involves a series of adhesive interactions between leukocytes and the vascular endothelium (Springer, 1994; Butcher and Picker, 1996). In some cases, initial binding of leukocyte adhesion molecules to their endothelial ligands can lead to the transient arrest, or tethering, of the leukocyte followed by leukocyte rolling. Rolling cells can then be "activated" via incompletely understood mechanisms, which lead to an increase in the activity of certain adhesion molecules and the arrest of the leukocyte on the luminal surface of the endothelium. In other cases, leukocytes may arrest immediately, without rolling. After arrest, leukocytes can extravasate into the underlying tissue. Different leukocyte adhesion molecules are used for different steps in this process (von Andrian et al., 1991). L-selectin and the E- and P-selectin ligands are expressed on some leukocytes and mediate initial adhesion (tethering and rolling), but do not support firm arrest. In contrast, the leukocyte  $\beta 2$  integrins  $\alpha L\beta 2$  (LFA-1, CD11a/CD18) and

$\alpha M\beta 2$  (Mac-1, CD11b/CD18) mediate firm arrest but not initial adhesion. Another integrin subfamily, the  $\alpha 4$  integrins  $\alpha 4\beta 1$  (VLA-4, CD49d/CD29) and  $\alpha 4\beta 7$  (LPAM-1), can support both initial adhesion and firm arrest (Sriramarao et al., 1994; Alon et al., 1995; Berlin et al., 1995).

Presentation of certain leukocyte adhesion molecules on microvilli substantially enhances the ability of these molecules to support tethering and rolling on endothelial ligands. The importance of receptor distribution was highlighted by studies of the adhesion molecules L-selectin and CD44 (von Andrian et al., 1995). L-selectin is located primarily on microvilli, whereas CD44 is concentrated on the planar cell body. L-selectin-CD44 chimeras were used to examine the role of cytoplasmic and transmembrane domains in receptor localization and the ability to roll on ligands. A chimera comprising the L-selectin extracellular domain fused to the CD44 transmembrane and cytoplasmic domains (L/CD44) localized to the cell body, and a CD44 extracellular, L-selectin transmembrane and cytoplasmic domain chimera (CD44/L) localized to microvilli. Although replacement of the transmembrane and cytoplasmic domains of L-selectin or CD44 did not alter their ability to adhere under static (no flow) conditions, it affected adhe-

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sion under flow. L-selectin (on microvilli) supported initial attachment better than the L/CD44 chimera (cell body), whereas CD44/L (microvilli) supported initial attachment better than CD44 (cell body). These results indicate that the transmembrane and/or cytoplasmic domains account for the differences in localization of L-selectin and CD44, and strongly suggest that microvillous localization is important for optimal initial adhesion under flow.

Available evidence about the distribution of integrins on the leukocyte surface is also consistent with a role for microvillous presentation in initial adhesion under flow. Integrins  $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2 are concentrated on the planar cell body and do not support initial adhesion, whereas  $\alpha$ 4 $\beta$ 1 and  $\alpha$ 4 $\beta$ 7 localize primarily to microvilli and do support tethering and rolling (Erlandsen et al., 1993; Berlin et al., 1995). The mechanism underlying the differential topography of these integrins on nonadherent leukocytes is not known. However, studies of other integrins have established a central role for the cytoplasmic domains of integrin  $\beta$  subunits in localization of integrins on membranes of adherent cells (LaFlamme et al., 1992, 1994; Briesewitz et al., 1993; Sastry and Horwitz, 1993; Ylanne et al., 1993; Pasqualini and Hemler, 1994). Interactions with several cytoskeletal proteins such as talin and  $\alpha$ -actinin (demonstrated *in vitro*) are suggestive of links to microfilament fibers that may regulate protein localization. In addition, several transmembrane proteins, such as tetraspan proteins (including CD9, CD53, CD63, CD81, and CD82) (Slupsky et al., 1989; Rubinstein et al., 1994; Berditchevski et al., 1995, 1996, 1997), CD32 (Fc $\gamma$ RIIA) (Worth et al., 1996), and CD47 (integrin-associated protein) (Lindberg et al., 1993), have been shown to associate with integrins. These interactions are known or suspected to involve the extracellular domains of these proteins, and their role (if any) in integrin localization is unknown.

We sought to examine the role of leukocyte integrin transmembrane and cytoplasmic domains in microvillous localization and in initial adhesion under flow. Here we report that replacement of  $\alpha$ 4 $\beta$ 7 transmembrane and cytoplasmic domains with the homologous domains of  $\beta$ 2 integrins does not alter membrane localization or initiation of adhesion under flow. This unexpected result suggests that differences in localization of leukocyte integrins to microvilli are determined by the extracellular domain.

## Materials and Methods

### Cell Lines

K562 human erythroleukemia cells (CCL 243; American Type Culture Collection, Rockville, MD) were maintained in growth medium: RPMI 1640 supplemented with 10% FBS, penicillin (50 IU/ml), streptomycin (50  $\mu$ g/ml), and glutamine (2 mM). Stably transfected K562 lines expressing human integrin  $\alpha$ 4 (K562- $\alpha$ 4 $\beta$ 1) and  $\alpha$ 4 $\beta$ 7 (K562- $\alpha$ 4 $\beta$ 7) were described previously (Tidswell et al., 1997). Additional K562 integrin transfectants were provided by other investigators: K562- $\alpha$ L $\beta$ 2 and K562- $\alpha$ M $\beta$ 2 (I. Graham, Washington University, St. Louis, MO) (Graham et al., 1994); K562- $\alpha$ 6 $\beta$ 1 (A. Sonnenberg, The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Hogervorst et al., 1993); K562- $\alpha$ v $\beta$ 3 (S. Blystone, Washington University) (Blystone et al., 1994).

### cDNAs

The pCDM8-integrin  $\alpha$ 4 cDNA plasmid (Kamata et al., 1995) was a gift from Y. Takada (Scripps Research Institute, La Jolla, CA). The cloning of

the  $\beta$ 7 cDNA has been previously described (Erle et al., 1991). Integrin  $\beta$ 2 cDNA (Hickstein et al., 1988) was provided by D. Hickstein (University of Washington, Seattle, WA). Integrin  $\alpha$ L (Larson et al., 1989) and  $\alpha$ M (Corbi et al., 1988) cDNAs were provided by T. Springer (Center for Blood Research, Boston, MA).  $\alpha$ E cDNA (Shaw et al., 1994) was a gift from G. Russell and M. Brenner (Harvard Medical School, Boston, MA). Chimeric integrin subunits were constructed using splice overlap extension PCR (Horton et al., 1989). The amino acid splice sites for each construct are shown in Fig. 1. Chimeric  $\alpha$  subunits were subcloned into pCDM8 (Invitrogen, San Diego, CA) and chimeric  $\beta$  subunits were subcloned into pCEP4 (Invitrogen). The integrity of the constructs was confirmed by DNA sequencing.

### Transfections and Expression

K562 cells in log phase growth were washed twice in electroporation buffer (HBSS, 20 mM Hepes, 6 mM dextrose).  $8 \times 10^6$  cells were resuspended in 0.2 ml buffer and transferred to 2 mm electroporation cuvettes (BTX, San Diego, CA). Stable transfections were performed using the pCDM8- $\alpha$ 4 and pCEP- $\beta$ 7 constructs (25  $\mu$ g each) plus 5  $\mu$ g pBK-neo (Stratagene, La Jolla, CA) at 900  $\mu$ Farad, 200 V, 13  $\Omega$  (Electro Cell Manipulator 600; BTX). Samples were left at room temperature for 10 min before and after electroporation. Cells were then placed in 20 ml growth medium and maintained at 37°C in a 5% CO<sub>2</sub> incubator. After 48 h, transfectants were selected using growth medium containing 500  $\mu$ g/ml each of Hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) and G418 (GIBCO BRL, Gaithersburg, MD). Stably transfected clones were obtained by limiting dilution and analyzed by flow cytometry as previously described (Tidswell et al., 1997). Transfectants were maintained in selection medium.

### Antibodies

Fib 504 (anti-integrin  $\beta$ 7) was a gift of E.C. Butcher (Stanford University, Palo Alto, CA) (Andrew et al., 1994). HP1/2 was used to detect the integrin  $\alpha$ 4 subunit (Pulido et al., 1991). 7E4 (anti- $\beta$ 2) and GoH3 (anti- $\alpha$ 6) were purchased from Immunotech (Westbrook, ME). L230 (anti- $\alpha$ v) and B11G2 (anti- $\alpha$ 5) were provided by D. Sheppard and C. Damsky (University of California, San Francisco, CA). Antibodies against the integrin-associated proteins CD53 (clone HI29; PharMingen, San Diego, CA), CD63 (MAB1787; Chemicon International, Inc., Temecula, CA), and CD32 (clone IV.3; Medarex, East Annandale, NJ) were obtained from commercial sources. The anti-CD47 antibody B6H12 was a gift from E. Brown (Washington University). Hybridoma supernatants or purified IgG preparations were used for flow cytometry analysis and immunoelectron microscopy. Gold-conjugated secondary antibodies (6- or 12-nm particles) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

### Immunoelectron Microscopy

Transfectants were immunolabeled in suspension. Cells were prefixed for 20 min in 0.2% paraformaldehyde/PBS at 4°C. After washing twice with PBS containing 10% goat serum, cells were incubated with primary antibody diluted in PBS containing 10% goat serum. After a 30-min incubation at 4°C and a wash with PBS, gold-conjugated secondary antibody in PBS was added. Cells were incubated for 30 min at 4°C, washed twice with PBS, and fixed in 0.1 M sodium cacodylate buffer, pH 7.4, with 3% glutaraldehyde. Before embedding, cells were rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide/0.1 M cacodylate buffer. After rinsing, cells were dehydrated through a graded series of acetone washes, and infiltrated and embedded in Spurr's epoxy resin (Ted Pella, Inc., Redding, CA). Sections (70-nm thick) were stained with uranyl acetate and lead citrate, and examined with a CM120 Phillips electron microscope (Phillips Electron Optics, Inc., Mahwah, NJ). 50–100 cells were examined and representative cells were photographed. Each experiment was repeated at least twice. Colloidal gold distribution on immunolabeled cells was determined by analysis of electron micrographs ( $\times 19,500$ – $\times 40,000$ ). Gold particles associated with cell body or microvilli were counted from 3–11 micrographs that represented different individual cells.

### Static Adhesion Assay

Static adhesion assays were performed as previously described (Tidswell et al., 1997). Briefly, 21-well glass slides (Structure Probe, West Chester, PA) were coated with mucosal addressin cell adhesion molecule-1 (MAD-

CAM-1)<sup>1</sup>-IgG fusion protein (4 ng/well) or intercellular adhesion molecule-1 (ICAM-1)-C $\kappa$  fusion protein (0.23  $\mu$ g/well) overnight at 4°C. MAdCAM-1-IgG (Tidswell et al., 1997) was a gift of M. Briskin (Leukocyte Inc., Boston, MA). ICAM-1-C $\kappa$  (Piali et al., 1995) was a gift of B. Imhof (Centre Medicale Universitaire, Geneva, Switzerland). After blocking with 4% BSA for 2 h,  $4 \times 10^4$  cells were resuspended in 15  $\mu$ l of 10 mM Hepes, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, with or without 1 mM MnCl<sub>2</sub>, and added to the wells. Cells were allowed to adhere for 90 min at room temperature. After washing, cells were fixed with 3% glutaraldehyde and stained with 0.5% crystal violet. Adherent cells were counted using a microscope. Assays were performed in triplicate.

### Adhesion under Flow

Capillary tubes (100- $\mu$ l capacity; Drummond, Broomall, PA) were coated at 4°C overnight with 20  $\mu$ l of solution containing MAdCAM-IgG (0.2  $\mu$ g/ml) or ICAM-1-C $\kappa$  (0.7 mg/ml) and blocked with 4% BSA for 2 h at 37°C. K562 transfectants were washed with HBSS containing 1 mM EDTA, and resuspended at  $5 \times 10^5$  cells/ml in HBSS/10 mM Hepes containing 1 mM each of Ca<sup>2+</sup> and Mg<sup>2+</sup>. To measure adhesion under flow, cells were perfused through the coated capillary tube using a syringe pump (74900 series; Cole-Parmer Instrument Co., Vernon Hills, IL) at a flow rate of 0.67 ml/min. Calculated shear stress was 1.0 dyne/cm<sup>2</sup> according to Pousille's law of dynamic shear (Berlin et al., 1995). Results were captured using a TMS microscope (Nikon, Garden City, NJ), CCD video camera (Sony, Park Ridge, NJ), and time lapse SVMS videocassette recorder (Panasonic, Secaucus, NJ). After 2 min of flow, five randomly chosen fields from the coated region were analyzed for 5 s each. All adherent cells (rolling or arrested) were counted. Cells did not adhere to areas coated with 4% BSA alone (control).

## Results

### Generation of Transfectants Expressing Chimeric and Mutant $\alpha 4\beta 7$ Constructs

To examine the role of cytoplasmic and transmembrane domains of  $\alpha 4\beta 7$  in adhesion and membrane localization, we expressed chimeric integrin heterodimers (Fig. 1). Each construct included the extracellular domains of  $\alpha 4$  and  $\beta 7$ . In one construct, designated  $\alpha 4\beta 7(\alpha L\beta 2c)$ , most of the cytoplasmic domains of  $\alpha 4$  and  $\beta 7$  were replaced with homologous regions of  $\alpha L$  and  $\beta 2$ . In a second construct,  $\alpha 4\beta 7(\alpha M\beta 2tc)$ , all of the transmembrane and cytoplasmic domains of  $\alpha 4$  and  $\beta 7$  were replaced with homologous domains of  $\alpha M$  and  $\beta 2$ . The integrin  $\alpha$  and  $\beta$  subunit cDNAs were cotransfected into K562 human erythroleukemia cells, which do not normally express  $\alpha 4$ ,  $\alpha L$ ,  $\alpha M$ ,  $\beta 2$ , or  $\beta 7$ . The levels of protein expression on the transfectants K562- $\alpha 4\beta 7$ , K562- $\alpha 4\beta 7(\alpha L\beta 2c)$ , and K562- $\alpha 4\beta 7(\alpha M\beta 2tc)$  were determined to be similar by flow cytometry (Fig. 2, A-C). Two truncated cDNAs,  $\alpha 4\Delta$  (truncated after amino acids GFFKR) and  $\beta 7\Delta$  (truncated after amino acids VLAYR), were also produced. The  $\alpha 4\Delta$  was expressed in combination with  $\beta 7$  on transfected K562 cells (K562- $\alpha 4\Delta\beta 7$ ), although at levels somewhat below those seen with other constructs (data not shown). We were unable to detect expression of  $\beta 7\Delta$  on cells cotransfected with  $\alpha 4$ , despite a previous report that the homologous truncation mutant of mouse  $\beta 7$  was expressed on transfected cells (Crowe et al., 1994). We were able to document heterologous expression of other wild-type integrins, including  $\alpha L\beta 2$ ,  $\alpha M\beta 2$ ,  $\alpha 6\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha E\beta 7$ , on appropriate K562 transfectants by flow cytometry (Fig. 2, D-H).

1. Abbreviations used in this paper: ICAM-1, intercellular adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

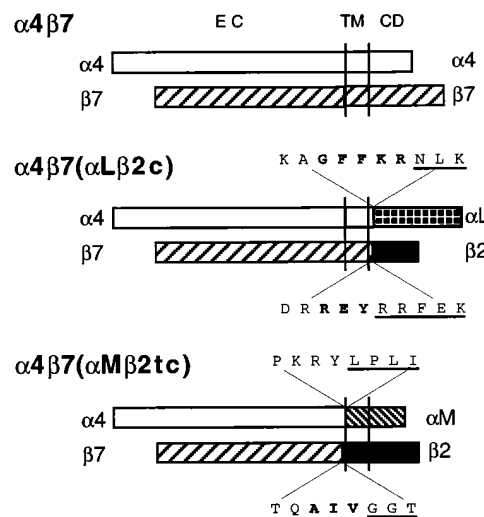


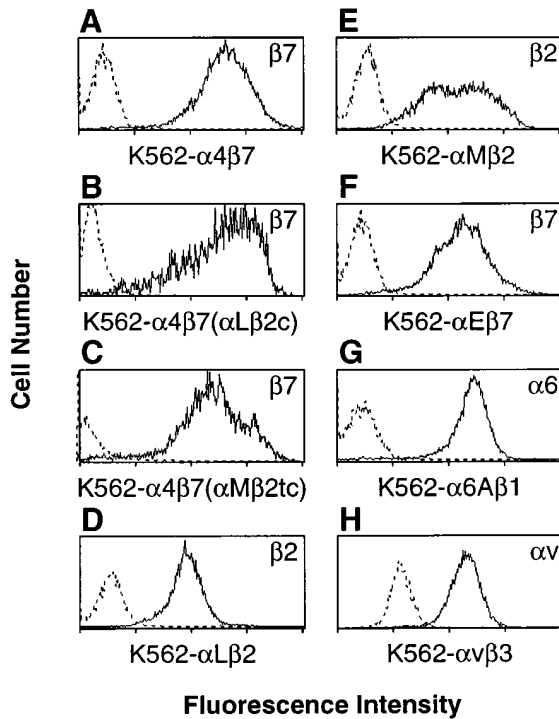
Figure 1. Schematic representation of wild-type and chimeric  $\alpha 4\beta 7$  integrins. The amino acid sequence at the splice site is shown with conserved regions in bold, and the  $\alpha L\beta 2$  or  $\alpha M\beta 2$  sequences underlined.

### Adhesion of Transfectants to MAdCAM-1 under Static Conditions

We analyzed the ability of integrin-transfected K562 cells to adhere to MAdCAM-1, an  $\alpha 4\beta 7$  ligand, and ICAM-1, an  $\alpha L\beta 2$  ligand, under static (no flow) conditions (Fig. 3). After transfection with  $\alpha 4$  cDNA alone, K562 cells express  $\alpha 4\beta 1$  but not  $\alpha 4\beta 7$  (Tidswell et al., 1997). These cells failed to adhere to MAdCAM-1. In contrast, cells transfected with both  $\alpha 4$  and  $\beta 7$  (K562- $\alpha 4\beta 7$ ) adhered efficiently to MAdCAM-1. As previously reported, adhesion was increased in the presence of Mn<sup>2+</sup>. The chimeric transfectants, K562- $\alpha 4\beta 7(\alpha L\beta 2c)$  and K562- $\alpha 4\beta 7(\alpha M\beta 2tc)$ , also adhered to MAdCAM-1, and the extent of adhesion was very similar for chimeric and wild-type  $\alpha 4\beta 7$  transfectants. As expected, K562- $\alpha 4\beta 7$  cells did not adhere to ICAM-1, whereas K562- $\alpha L\beta 2$  cells did.

### Adhesion of Transfectants to MAdCAM-1 under Flow

We next examined the ability of the wild-type and chimeric integrins to initiate adhesion under flow (Fig. 4). K562- $\alpha 4\beta 7$  cells adhered to MAdCAM-1 under flow. This adhesion was dependent upon both  $\alpha 4\beta 7$  and MAdCAM-1 because K562 cells transfected with  $\alpha 4$  alone (K562- $\alpha 4\beta 1$ ) did not adhere to MAdCAM-1, and K562- $\alpha 4\beta 7$  cells did not adhere to capillary tubes coated with other ligands, such as ICAM-1. The chimeric integrins,  $\alpha 4\beta 7(\alpha L\beta 2c)$  and  $\alpha 4\beta 7(\alpha M\beta 2tc)$ , both supported adhesion to MAdCAM-1 under flow. Wild-type  $\alpha 4\beta 7$  and the chimeric integrins were very similar in their ability to initiate adhesion under flow. We also examined the resistance to detachment from MAdCAM-1 at increasing shear stress conditions (up to 10 dynes/cm<sup>2</sup>). We did not find differences between wild-type and chimeric transfectants in this assay (data not shown). At a shear stress of 1 dyne/cm<sup>2</sup>, most cells remained adherent during the time interval of cell counts. Transfectants expressing a truncated  $\alpha 4$  subunit (K562- $\alpha 4\Delta\beta 7$ ) also adhered to MAdCAM-1 under flow, although at a somewhat lower rate (perhaps related to lower levels

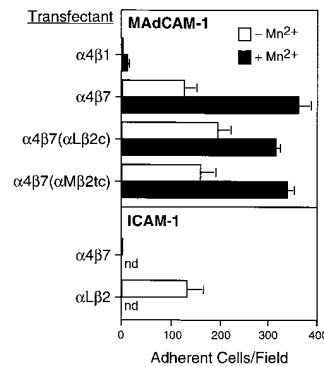


**Figure 2.** Cell surface expression of wild-type and chimeric integrins. K562- $\alpha 4\beta 7$  (A), K562- $\alpha 4\beta 7$  ( $\alpha L\beta 2c$ ) (B), and K562- $\alpha 4\beta 7$  ( $\alpha M\beta 2tc$ ) (C) cells were stained with the anti- $\beta 7$  antibody, Fib 504, as shown. Each of these three transfectants was also recognized by other antibodies specific for the  $\alpha 4$  subunit or the  $\alpha 4\beta 7$  heterodimer, but were not recognized by anti- $\alpha E$  antibodies (not shown). K562- $\alpha L\beta 2$  were recognized by antibodies to  $\beta 2$  (D) and  $\alpha L$  (not shown). K562- $\alpha M\beta 2$  cells were recognized by antibodies to  $\beta 2$  (E) and  $\alpha M$  (not shown). K562- $\alpha E\beta 7$  cells stained with antibodies to  $\beta 7$  (F) and  $\alpha E$ , but not with anti- $\alpha 4$  antibodies (not shown). K562- $\alpha 6A\beta 1$  cells were stained with the anti- $\alpha 6$  antibody GoH3 (G). There was low level expression of  $\alpha V$  on nontransfected K562 cells, and higher expression on K562- $\alpha V\beta 3$  cells as determined using the anti- $\alpha V$  antibody L230 (H). Fluorescence intensity is shown on a log scale (one log per division). Dotted and solid histograms represent staining with nontransfected and transfected K562 cells, respectively.

of expression of this construct, data not shown). As expected from previous reports (von Andrian et al., 1991), K562- $\alpha L\beta 2$  cells were unable to initiate adhesion to their ligand, ICAM-1, under flow (Fig. 4).

#### Membrane Localization of Wild-type and Chimeric $\alpha 4\beta 7$ , $\alpha L\beta 2$ , and $\alpha M\beta 2$ Integrins on Transfected K562 Cells

Previous reports demonstrated that integrin  $\alpha 4\beta 7$  localizes primarily to microvilli of mouse TK-1 lymphoma cells, whereas integrins  $\alpha L\beta 2$  and  $\alpha M\beta 2$  localize primarily to the cell body of TK-1 cells and human neutrophils respectively (Erlandsen et al., 1993; Berlin et al., 1995). We began by using immunoelectron microscopy to determine whether these integrins would localize similarly in transfected K562 cells. K562 cells had numerous microvillous projections.  $\alpha 4\beta 7$  was found primarily on microvilli (Fig. 5 A). In contrast,  $\alpha L\beta 2$  and  $\alpha M\beta 2$  integrins were located primarily on the cell body (Fig. 5, B–D). Both  $\alpha 4\beta 7$  ( $\alpha L\beta 2c$ ) and

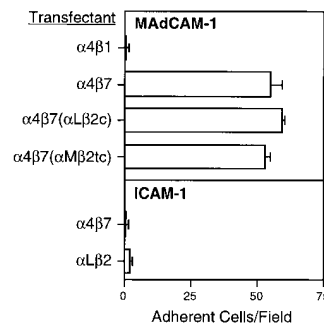


**Figure 3.** Static adhesion of transfectants to immobilized ligands. Adhesion of various integrin transfectants to MAdCAM-1 (top) and ICAM-1 (bottom) was measured in the presence and absence of  $Mn^{2+}$ . Bars indicate SEM. nd, not determined.

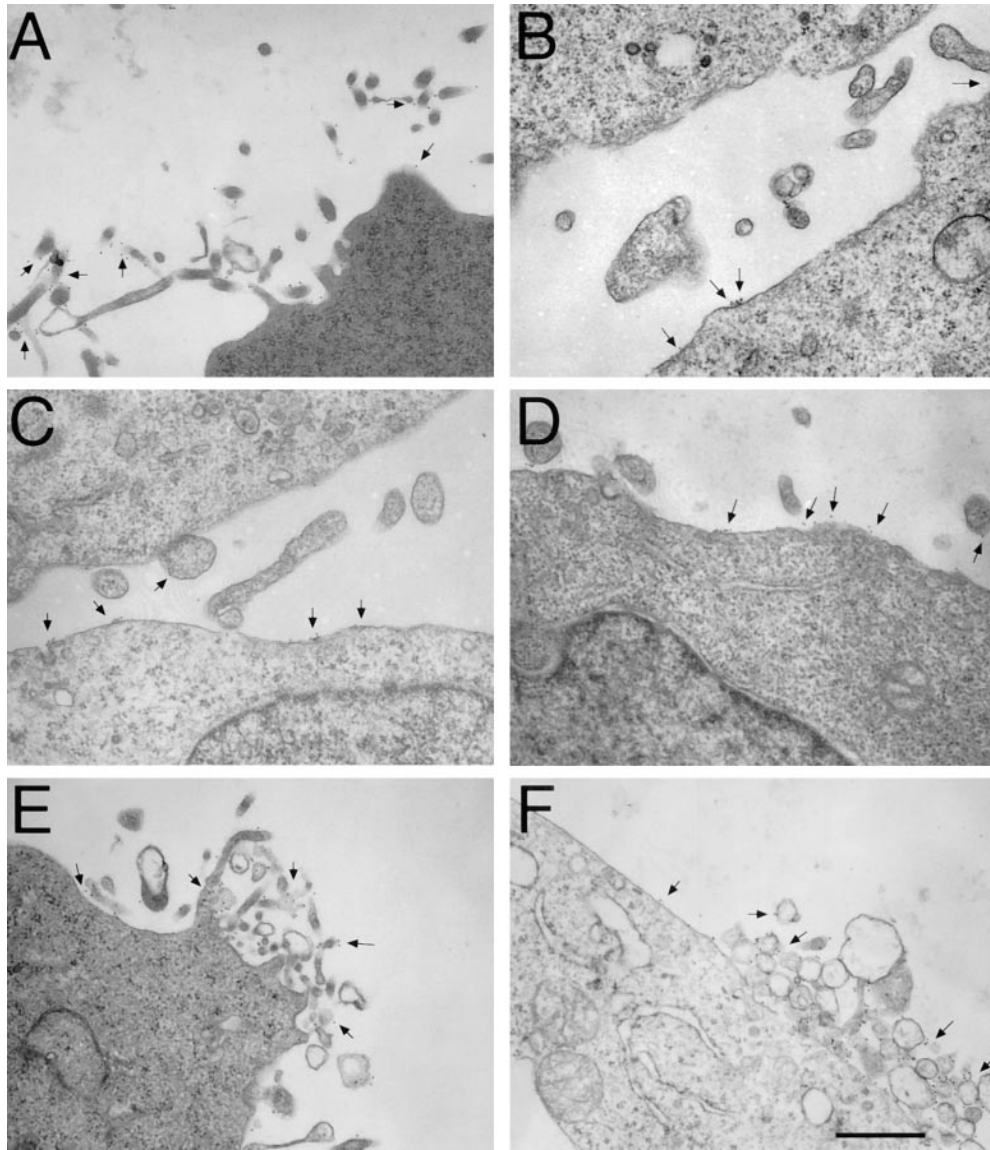
$\alpha 4\beta 7$  ( $\alpha M\beta 2tc$ ) localized to microvillous projections (Fig. 5, E and F). A quantitative analysis of the distributions of these integrins is shown in Table I. The  $\alpha 4\Delta\beta 7$  construct was also concentrated on microvilli (not shown). These results indicate that the transmembrane and cytoplasmic domains are not responsible for the differential localization of  $\alpha 4\beta 7$  versus  $\alpha L\beta 2$  and  $\alpha M\beta 2$ .

#### Presentation of Other Leukocyte Integrins on Microvilli

In addition to  $\alpha 4\beta 7$ ,  $\alpha L\beta 2$ , and  $\alpha M\beta 2$ , leukocytes express other integrins that play roles in adhesion to endothelial cells, to other cells, and to extracellular matrix proteins. One of these,  $\alpha 4\beta 1$ , can initiate adhesion to vascular cell adhesion molecule-1 (VCAM-1) under flow and has been reported to be expressed on lymphocyte microvilli (Alon et al., 1995; Berlin et al., 1995). We confirmed that  $\alpha 4\beta 1$  was also expressed preferentially on microvilli of K562- $\alpha 4\beta 1$  transfectants (Table I). We found that the T cell integrin  $\alpha E\beta 7$  (Cepek et al., 1994), which can mediate adhesion to epithelium but has no established role in endothelial adhesion, was also localized to microvilli of K562- $\alpha E\beta 7$  cells (Fig. 6 A, and Table I). Integrin  $\alpha 6A\beta 1$ , a laminin receptor which is expressed on monocytes and some lymphocytes, was concentrated on microvilli of K562 transfectants (Fig. 6 B, and Table I). The vitronectin receptor, integrin  $\alpha V\beta 3$ , is also a receptor for the endothelial cell ligand platelet/endothelial cell adhesion molecule-1 (Piali et al., 1995) and is expressed on monocytes and other cells. We found that  $\alpha V\beta 3$  localized predominantly to microvilli (Fig. 6 C, and Table I). The fibronectin receptor, integrin  $\alpha 5\beta 1$ , is constitutively expressed on K562 cells and was also expressed predominantly on microvilli (not shown).



**Figure 4.** Adhesion to ligands under flow. Adhesion of various integrin transfectants to MAdCAM-1 (top) and ICAM-1 (bottom) measured at a wall shear stress of 1 dyne/cm<sup>2</sup> in the absence of  $Mn^{2+}$ , as described in Materials and Methods. Bars indicate SEM.



**Figure 5.** Localization of  $\alpha 4\beta 7$ ,  $\alpha L\beta 2$ ,  $\alpha M\beta 2$ , and chimeric integrins by immuno-electron microscopy. K562 transfectants were stained for protein expression using 12-nm gold particles (arrows) as described in Materials and Methods. Wild-type  $\alpha 4\beta 7$  (identified using the anti- $\beta 7$  antibody, Fib 504) was localized predominantly to microvilli of K562- $\alpha 4\beta 7$  transfectants (A). K562- $\alpha L\beta 2$  (B and C) and K562- $\alpha M\beta 2$  (D) were stained with antibodies to  $\alpha L$  (B) or  $\beta 2$  (C and D), demonstrating that  $\alpha L\beta 2$  and  $\alpha M\beta 2$  were expressed mostly on the cell body. K562 cells transfected with the chimeric integrins  $\alpha 4\beta 7(\alpha L\beta 2c)$  (E) and  $\alpha 4\beta 7(\alpha M\beta 2c)$  (F) were stained with Fib 504. These chimeric integrins were found predominantly on microvilli. Photomicrographs are representative of integrin distribution on the 50–100 cells examined in each sample. Bar, 0.5  $\mu m$ .

### Localization of Transmembrane Proteins Known to Associate with Integrins

Integrins have been shown to associate with a variety of intracellular and transmembrane proteins. Some of these interactions occur within the cell and are mediated by integrin cytoplasmic domains, whereas others are known or suspected to be extracellular. Since our results suggested that extracellular (and not transmembrane or cytoplasmic) domains determine integrin localization, we performed immunoelectron microscopy to localize several cell surface proteins known or suspected to interact with integrin extracellular domains. Several members of the tetraspan family of transmembrane proteins have been shown to associate with  $\alpha 4\beta 1$ ,  $\alpha 4\beta 7$ ,  $\alpha 6\beta 1$ , and some other integrins (Berdichevski et al., 1996; Mannion et al., 1996). These associations are likely to involve the extracellular domains of tetraspans and integrins (see Discussion). Several tetraspans are constitutively expressed on K562 cells. CD53 ( $82 \pm 5\%$  on microvilli, Fig. 7 A), CD63 ( $92 \pm 4\%$  on microvilli, Fig. 7 B), and CD81 and CD82 (data not shown)

are all localized predominantly to microvilli. Another transmembrane protein, CD47 (integrin-associated protein), has been shown to associate with  $\alpha v\beta 3$  via its extracellular domain. CD47, like  $\alpha v\beta 3$ , was distributed mostly on microvilli (Fig. 7 C). CD32 is a transmembrane protein that has been reported to associate with  $\alpha M\beta 2$  (which is on the cell body; Fig. 5 C) and possibly with the tetraspan CD82 (found on microvilli, see above) (Lebel-Binay et al., 1995). CD32 was expressed on both microvilli ( $62 \pm 15\%$ ) and the cell body ( $38 \pm 15\%$ ) (Fig. 7 D).

### Discussion

In this study, we examined the role of leukocyte integrin cytoplasmic and transmembrane domains in adhesion under flow and microvillous localization. We began by confirming that previously described differences in leukocyte integrin adhesive activity and membrane localization were also seen in K562 cell integrin transfectants. As expected,  $\alpha 4\beta 7$  was able to initiate adhesion under flow and localized

Table I. Integrin Distribution on Transfected K562 Cells

| Integrin                             | Percent on microvilli | Percent on body | Gold particles counted |
|--------------------------------------|-----------------------|-----------------|------------------------|
| $\alpha 4\beta 7$                    | $89 \pm 9$            | $11 \pm 9$      | 1,288                  |
| $\alpha 4\beta 1$                    | $81 \pm 7$            | $19 \pm 7$      | 348                    |
| $\alpha L\beta 2$                    | $31 \pm 14$           | $69 \pm 14$     | 99                     |
| $\alpha M\beta 2$                    | $22 \pm 13$           | $78 \pm 13$     | 236                    |
| $\alpha 4\beta 7(\alpha L\beta 2c)$  | $90 \pm 9$            | $10 \pm 9$      | 260                    |
| $\alpha 4\beta 7(\alpha M\beta 2tc)$ | $87 \pm 6$            | $13 \pm 6$      | 386                    |
| $\alpha E\beta 7$                    | $83 \pm 6$            | $17 \pm 6$      | 149                    |
| $\alpha 6\beta 1$                    | $77 \pm 9$            | $23 \pm 9$      | 401                    |
| $\alpha v\beta 3$                    | $80 \pm 10$           | $20 \pm 10$     | 2,741                  |

Transfected K562 cells expressing various integrins were analyzed by immunoelectron microscopy after staining with appropriate anti-integrin antibodies. For each integrin, all gold particles in at least three photomicrographs were counted. Percentages are presented as mean  $\pm$  SD.

to microvilli, whereas  $\alpha L\beta 2$  and  $\alpha M\beta 2$  mediated adhesion only under static conditions and localized to the planar cell body. Two chimeras,  $\alpha 4\beta 7(\alpha L\beta 2c)$  and  $\alpha 4\beta 7(\alpha M\beta 2tc)$ , were expressed to examine the roles of the extracellular, transmembrane, and cytoplasmic domains in adhesion and membrane localization. Both chimeras were as efficient in initiating adhesion under flow as the wild-type integrin  $\alpha 4\beta 7$ . The chimeras were found predominantly on microvilli, indicating that the extracellular domain (and not the transmembrane or cytoplasmic domains) determined membrane localization of  $\alpha 4\beta 7$ . We have so far been unable to determine whether  $\beta 2$  integrin localization to the cell body is also independent of the transmembrane and cytoplasmic domains. We attempted to address this issue by expressing a chimeric integrin composed of the extracellular domain of  $\alpha M\beta 2$  and the transmembrane and cytoplasmic domains of  $\alpha 4\beta 7$ , but have not yet been successful in these experiments. In addition to  $\alpha 4\beta 7$ , several other integrins ( $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha E\beta 7$ ) were localized to microvilli. Transmembrane proteins known or suspected to interact with integrin extracellular domains, including tetraspan family members and CD47, were also found to be concentrated on microvilli.

The ability of leukocyte adhesion molecules to support initial adhesion under flow is influenced by several factors including affinity, avidity, and accessibility to endothelial ligands. Previous studies of nonintegrin adhesion molecules indicate that cytoplasmic domains can have dramatic effects upon initiating adhesion under flow without altering static adhesion. Experiments involving the use of L-selectin-CD44 chimeras suggest that cytoplasmic domains may influence adhesion under flow by targeting these receptors to the microvillous or cell body (see Introduction). However, it is clear that the cytoplasmic domain of L-selectin also has effects on initiation of adhesion that are independent of receptor positioning. A truncation mutant of L-selectin lacking the 11 COOH-terminal amino acid residues of the cytoplasmic domain was localized to microvilli and retained the ability to bind ligand, but was unable to support rolling on endothelium (Kansas et al., 1993; Pavalko et al., 1995). This mutant lost the ability to associate with the cytoskeletal proteins  $\alpha$ -actinin and vinculin, suggesting that interactions between adhesion molecule cytoplasmic domains and cytoskeletal proteins can be important in regulation of initial adhesive interactions under flow. We

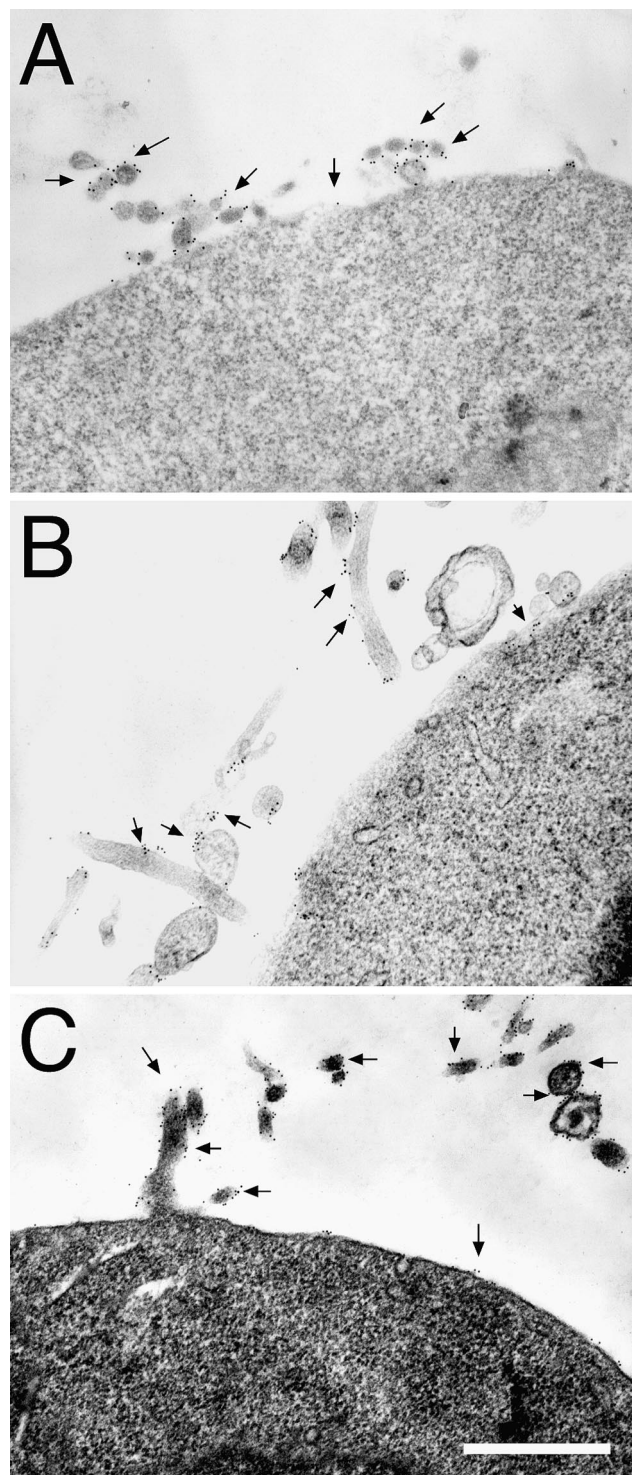
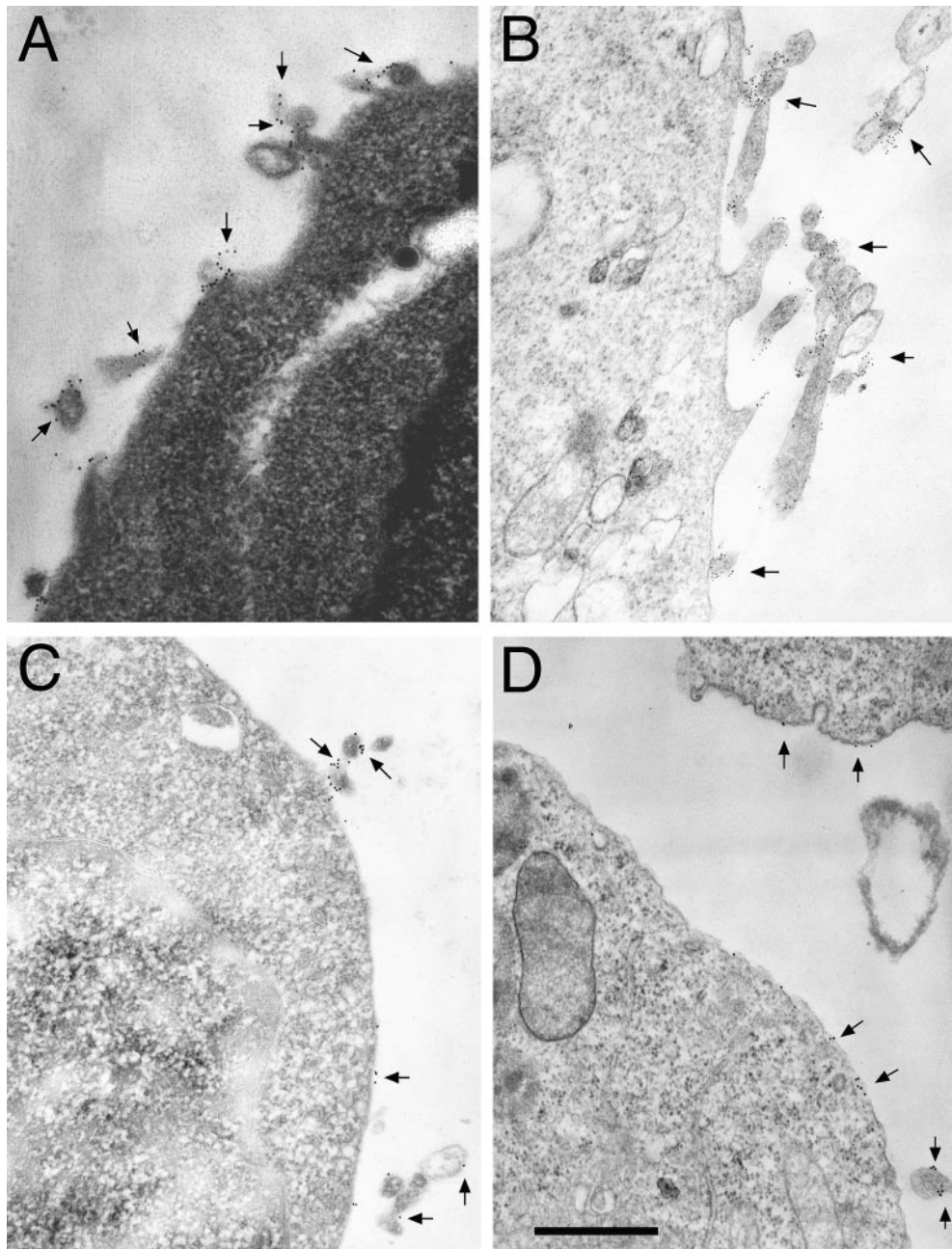


Figure 6. Localization of  $\alpha E\beta 7$ ,  $\alpha 6\beta 1$ , and  $\alpha v\beta 3$  integrins by immunoelectron microscopy. K562 transfectants were stained for protein expression using 12-nm gold particles (arrows) as described in Materials and Methods. Staining of K562- $\alpha E\beta 7$  with Fib 504 (anti- $\beta 7$ , A), K562- $\alpha 6\beta 1$  with anti- $\alpha 6$  (GoH3, B), and K562- $\alpha v\beta 3$  with L230 (anti- $\alpha v$ , C) revealed that each of these integrins was found primarily on microvilli. Bar, 0.5  $\mu m$ .





**Figure 7.** Localization of integrin-associated proteins by immunoelectron microscopy. The tetraspan proteins CD53 (A) and CD63 (B), the  $\alpha v\beta 3$ -associated protein CD47 (C), and CD32 (D) were localized by immunoelectron microscopy using 12-nm (A, C, and D) or 6-nm (B) gold particles (arrows). The tetraspan proteins and CD47 localized primarily to microvilli, whereas CD32 was found in substantial amounts on both the cell body and microvilli. Non-transfected K562 cells (A) and K562- $\alpha 4\beta 7$  (B), K562- $\alpha V\beta 3$  (C), and K562- $\alpha M\beta 2$  (D) transfectants were used for staining. Bar, 0.5  $\mu m$ .

found that replacement of the cytoplasmic and/or transmembrane domains of  $\alpha 4\beta 7$  (which does support adhesion under flow) with the homologous domains of  $\alpha L\beta 2$  or  $\alpha M\beta 2$  (which do not) did not affect adhesion to the  $\alpha 4\beta 7$  ligand MAdCAM-1 under either static or flow conditions. This indicates that the cytoplasmic domains cannot account for the differences in ability of these integrins to support adhesion under flow. We also found that truncation of the  $\alpha 4$  subunit after the conserved GFFKR motif had little if any effect on initiation of  $\alpha 4\beta 7$ -mediated adhesion to MAdCAM-1. Others have previously shown that the same truncation of  $\alpha 4$  did not affect the ability of  $\alpha 4\beta 1$  to initiate adhesion to its ligand, VCAM-1 (Kassner et al., 1995). The  $\alpha 4$  truncation was reported to decrease the cell's resistance to detachment from VCAM-1 in the face

of increasing shear force, suggesting that the  $\alpha$  subunit cytoplasmic domain plays a role in strengthening adhesion. We were unable to detect any difference in resistance to detachment between wild-type and chimeric  $\alpha 4\beta 7$  integrins, suggesting that  $\alpha 4\beta 7$ ,  $\alpha L\beta 2$ , and  $\alpha M\beta 2$  integrin cytoplasmic domains are similar in their ability to mediate adhesion strengthening.

Many of the adhesion molecules that initiate adhesion to endothelium under flow are concentrated on leukocyte microvilli. These include L-selectin (Picker et al., 1991; Erlandsen et al., 1993; Pavalko et al., 1995), P-selectin glycoprotein ligand-1 (Moore et al., 1995; Bruehl et al., 1997), and the integrins  $\alpha 4\beta 7$  and  $\alpha 4\beta 1$  (Berlin et al., 1995; and this report). Other adhesion molecules, including CD44 and the integrins  $\alpha L\beta 2$  and  $\alpha M\beta 2$ , are found predomi-



nantly on the cell body (Erlandsen et al., 1993; Berlin et al., 1995; von Andrian et al., 1995; and this report). Little information is available about the mechanisms that lead to the selective display of certain adhesion molecules on microvilli. It seems likely that interactions between the cytoplasmic domains of adhesion molecules and specific cytoskeletal elements can play an important role. In support of this concept, the localization of L-selectin-CD44 chimeras was shown to be determined by the cytoplasmic and/or transmembrane domains, and not by the extracellular domains (see Introduction). Concentration of integrins in other structures, such as focal adhesions and hemidesmosomes, is known to depend upon the  $\beta$  subunit cytoplasmic domain. We were surprised to find that our analysis of integrin chimeras did not demonstrate a role for the cytoplasmic or transmembrane domains of either the  $\alpha$  or  $\beta$  subunit in determining membrane localization. Replacement of the  $\alpha 4\beta 7$  cytoplasmic and/or transmembrane domains with homologous domains of  $\alpha L\beta 2$  or  $\alpha M\beta 2$  did not interfere with microvillous localization. Put another way, replacement of the extracellular domain of the  $\alpha L\beta 2$  or  $\alpha M\beta 2$  integrins with the extracellular domain of  $\alpha 4\beta 7$  resulted in a shift from cell body to microvillous localization. These results indicate an important role for the extracellular domain in directing localization of integrins to the microvillous versus the cell body.

Integrin extracellular domains can interact with other cell surface proteins. For example, the transmembrane protein CD47 interacts with  $\alpha v\beta 3$  and this interaction depends upon the extracellular domain of CD47 (Lindberg et al., 1996). Several members of the tetraspan family of transmembrane proteins, including CD53, CD63, CD81, and CD82, have been shown to coprecipitate with some integrins, including  $\alpha 4\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 4\beta 7$ , but not with  $\alpha L\beta 2$  or some other integrins (Berditchevski et al., 1996; Mannon et al., 1996). These interactions are likely to involve the integrin extracellular domain, since mutations of the  $\alpha 4$  subunit extracellular domain substantially reduce association whereas alterations of the  $\alpha$  subunit cytoplasmic domain have no effect. We found that CD47, CD53, CD63, CD81, and CD82, all known to associate with integrins that we localized to microvilli, were themselves concentrated on microvilli. Our data are consistent with the hypothesis that interactions with tetraspans and CD47 help target certain integrins to microvilli. The widespread expression of tetraspans and CD47 on leukocytes and other cells makes this hypothesis difficult to test directly. At least one integrin that we localized to microvilli,  $\alpha 5\beta 1$ , apparently does not associate with tetraspans or CD47, suggesting that other interactions also are important (Berditchevski et al., 1996). This hypothesis assumes that  $\alpha 4\beta 7$  and other microvillous integrins are actively concentrated on microvilli, but  $\alpha L\beta 2$  and  $\alpha M\beta 2$  are not. An alternative explanation of our results is that microvillous expression is the "default pathway" for integrins, and that the extracellular domains of  $\alpha L\beta 2$  and  $\alpha M\beta 2$  prevent these integrins from being displayed on microvilli. This could be mediated by interactions between  $\beta 2$  integrins and associated cell surface proteins. Although  $\alpha M\beta 2$  has been shown to associate with CD32, the pattern of expression of CD32 (on both cell body and microvilli) suggests that this interaction is not responsible for the concentration of  $\alpha M\beta 2$  on the cell body.

We found that many integrins and integrin-associated proteins were preferentially expressed on microvilli. Some of these integrins, including  $\alpha 4\beta 7$  and  $\alpha 4\beta 1$ , play important roles in mediating leukocyte adhesion under flow. Other microvillous integrins, such as  $\alpha 5\beta 1$  and  $\alpha E\beta 7$ , mediate adhesion to extracellular matrix proteins or epithelial cells, but have no known role in initiating leukocyte-endothelial interactions. This suggests that the assembly of multimolecular complexes containing integrins and integrin-associated proteins on microvilli may have other important roles in adhesion and signaling.

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